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The concerns of preimplantation genetic testing for aneuploidies: analysis and perspectives

Les préoccupations relatives aux tests génétiques préimplantatoires pour les aneuploïdies: analyse et perspectives

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Résumé. Les aneuploïdies chromosomiques affectent principalement les embryons provenant de patientes d'âge maternel avancé (AMA : > 35 ans), mêmes si environs 30 % des blastocystes obtenus chez de jeunes femmes pourraient être aneuploïdes. Pour éviter le risque de transfert d'embryons présentant une anomalie chromosomique (et/ou d'embryons atteints de maladies monogéniques dont les mutations causales ont été identifiées dans les génotypes parentaux), des tests génétiques pré-implantatoires (PGT) ont été introduits en fécondation *in vitro*. La biopsie du trophectoderme est le protocole le plus fiable et le plus validé pour récupérer un échantillon embryonnaire à cette fin. Cependant, pour que le PGT soit efficace sur le plan clinique, l'unité de FIV doit déjà maîtriser l'injection intracytoplasmique de spermatozoïde, la culture du blastocyste et la vitrification. Les embryologistes qualifiés doivent connaître toutes les forces et les pièges de chacune de ces techniques. De même, les cliniciens doivent clairement indiquer les limites techniques (*e.g.*, celles liées à la manipulation) et biologiques (*e.g.*, le mosaïcisme chromosomique) des tests génétiques quand ceux-là sont proposées aux patients.

Mots clés: biopsie du trophectoderme, blastocyste, vitrification, mosaïcisme chromosomique

Abstract. Chromosomal aneuploidies mainly affect embryos deriving from patients of advanced maternal age (AMA; >35 years), even if about 30% of the blastocysts obtained from young women might be aneuploid. To bypass the risk of transferring chromosomally-abnormal embryos (and/or embryos affected from monogenic diseases whose causative mutations were identified in the parental genotypes), pre-implantation genetic testing (PGT) has been introduced in ART. Trophectoderm biopsy is the most reliable and validated protocol to retrieve an embryonic specimen to this end. However, in order for PGT to be clinically-efficient, the IVF unit must be already proficient in ICSI, blastocyst culture and vitrification. Skilled embryologists should be familiar with all the strengths and pitfalls of each of these techniques. Similarly, the technical (e.g. manipulation-related hazards) and biological (e.g. chromosomal mosaicism) limitations of genetic testing should be clearly acknowledged from the practitioners, especially if counselling the couples indicated for PGT.

Key words : trophectoderm biopsy, blastocyst culture, vitrification, chromosomal mosaicism

In humans, the high incidence of aneuploidies derived from impaired meiosis in gametogenesis and/or mitosis during embryo preimplantation development is considered the single most impacting factor on embryo development, implantation failure and miscarriages. In advanced maternal age women (AMA defined as >35 years), the fall of fertility is mainly due to the exponential increasing incidence of embryonic aneuploidies. Indeed, in IVF, AMA shows an irrelevant impact upon fertilization rate, a mild impact upon embryo development but turns out to be dramatic for blastocysts chromosomal constitution. More than 90% of embryonic aneuploidies in embryos are imputable to full-chromosome constitutive impairments originated during oogenesis [1]. Conversely, structural chromosomal abnormalities seem mostly independent from maternal age and equally affect both the partners (e.g. segmental aneuploidies, copy number variations, micro-deletions and micro-duplication), therefore possibly arising from *de novo* events in oogenesis and spermatogenesis or mitosis [2].

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The clear association between increasing maternal age and decreasing success in conceiving both spontaneously and after IVF is becoming more and more relevant considering the worldwide trend in delaying the age at which women attempt to conceive. Several possible molecular and biochemical mechanisms involved in age-related infertility have been investigated in the effort to explain the reduced competence of aged oocytes. This ranged from premature separations of sister chromatids in meiosis I, mitochondrial disfunction, shortenings of telomeres, cohesions dysfunction and meiotic spindle abnormalities. However, none of them has been yet exhaustively elucidated and no related clinical therapy is available to counteract the fertility decline.

It is well-known that the incidence of aneuploidies is relatively low in new-born population (0.3%), and the main aneuploidies are trisomies for chromosomes 13, 18, 21 or sex chromosomes copy number variations, collectively causing more than 45% of all miscarriages. Recent evidences suggest that the incidence of aneuploidy reaches its highest levels after the first three days of preimplantation development, when no checkpoint against the occurrence of chromosomal abnormalities is in place [3, 4].

Chromosomal miss-segregation though represent an issue that is not limited to AMA patients: the baseline production of aneuploid blastocysts is 30% in women younger than 35 and increases to >90% in women older than 44 [1]. These are extremely relevant concepts, which are at the root of research efforts to sidestep the risk of transferring chromosomally abnormal embryos in couples undergoing IVF.

Pre-implantation genetic testing (PGT) was developed in the early 90s as a tool for embryo selection. It was aimed at identifying unaffected embryos from couples with specific monogenic diseases or structural chromosomal abnormalities that could be inherited by their offspring. It was introduced as a promising test, alternative to traditional prenatal diagnosis, and then also used to test embryos for aneuploidies.

The first version of PGT was based on blastomere biopsy at the cleavage stage, analysing nine chromosomes via fluorescent in situ hybridization (FISH). It failed to fulfil the promises of reducing both implantation failure per transfer and miscarriage rate [5]. Over time, challenging investigations outlined the reasons for such failure and set out to implement new approaches. FISH analysis was easily abandoned in favour of higher resolution and more accurate comprehensive chromosome testing (CCT) technologies like q-PCR, array-CGH, array-SNP, NGS. In contrast, the definition of the ideal biopsy stage was trickier. Throughout the last 30 years, three different settings were implemented: blastomere biopsy at the cleavage stage, polar bodies biopsy (from oocytes/zygotes) and trophectoderm biopsy from the blastocyst.

Cleavage stage biopsy and related concerns

The main workflow adopted across the years from the first theorization of PGT entailed the FISH-based analysis of one blastomere obtained from a cleavage stage embryo (day3 of preimplantation development) showing at least 6 blastomeres and less than 30% of fragmentation. Nevertheless, it has been demonstrated that this approach impacts embryo reproductive competence and viability [6]. It was in fact reported a striking 39% relative decrease in implantation rate of embryos submitted to blastomere biopsy with respect to sibling non-biopsied ones. As a consequence, the use of cleavage stage biopsy might result in a lower live birth rate (LBR) per intention to treat [5].

Moreover, the molecular methods for chromosomal testing are inefficient if adopted to analyse a single cell. Firstly, FISH is limited to the detection of only 9 chromosomes, while the whole set of 22 autosomes and 2 sex chromosomes might be impaired in preimplantation embryos. Secondly, even if the most advanced molecular techniques are adopted to test low input DNA samples, their accuracy might be undermined by technical artefacts resulting into higher rate of amplification failure and higher risk of inconclusive diagnosis [7]. Thirdly, cells might be in the S-phase of the cell cycle, therefore synthetizing their genome, which is a further source of false positive (FP) diagnoses [8]. Lastly, embryonic genome activation in humans does not occur before the 4 to 8 cell stage transition, therefore, in the first divisions after fertilization, there is no cell cycle control and the embryo is exposed to high risk of mitotic chromosomal miss-segregation [3]. Indeed, chromosomal mosaicism (i.e. the co-existence of cells with different chromosomal constitutions in the same embryo) reaches its highest incidence at this stage of development [4], which also represents the graveyard of most developmentally-incompetent (possibly mosaic) embryos (*figure 1*).

At present, cleavage stage biopsy is still widely used in conjunction with CCT platforms, but only one randomized controlled trial (RCT) has been published to verify the clinical effectiveness of this controversial setting [9].

Polar body biopsy and related concerns

Polar body (PB) biopsy entails the retrieval of both the first and second polar body from the oocyte/zygote. This approach is based on two premises: i) prenatal diagnosis after natural conception or molecular analysis of products of conception after miscarriage showed that vital trisomies mainly originate from an improper maternal meiosis, particularly in the first division [1]; ii) PBs are waste products of female meiosis and do not represent embryonic cells. For this reason, PB biopsy is still performed nowadays in

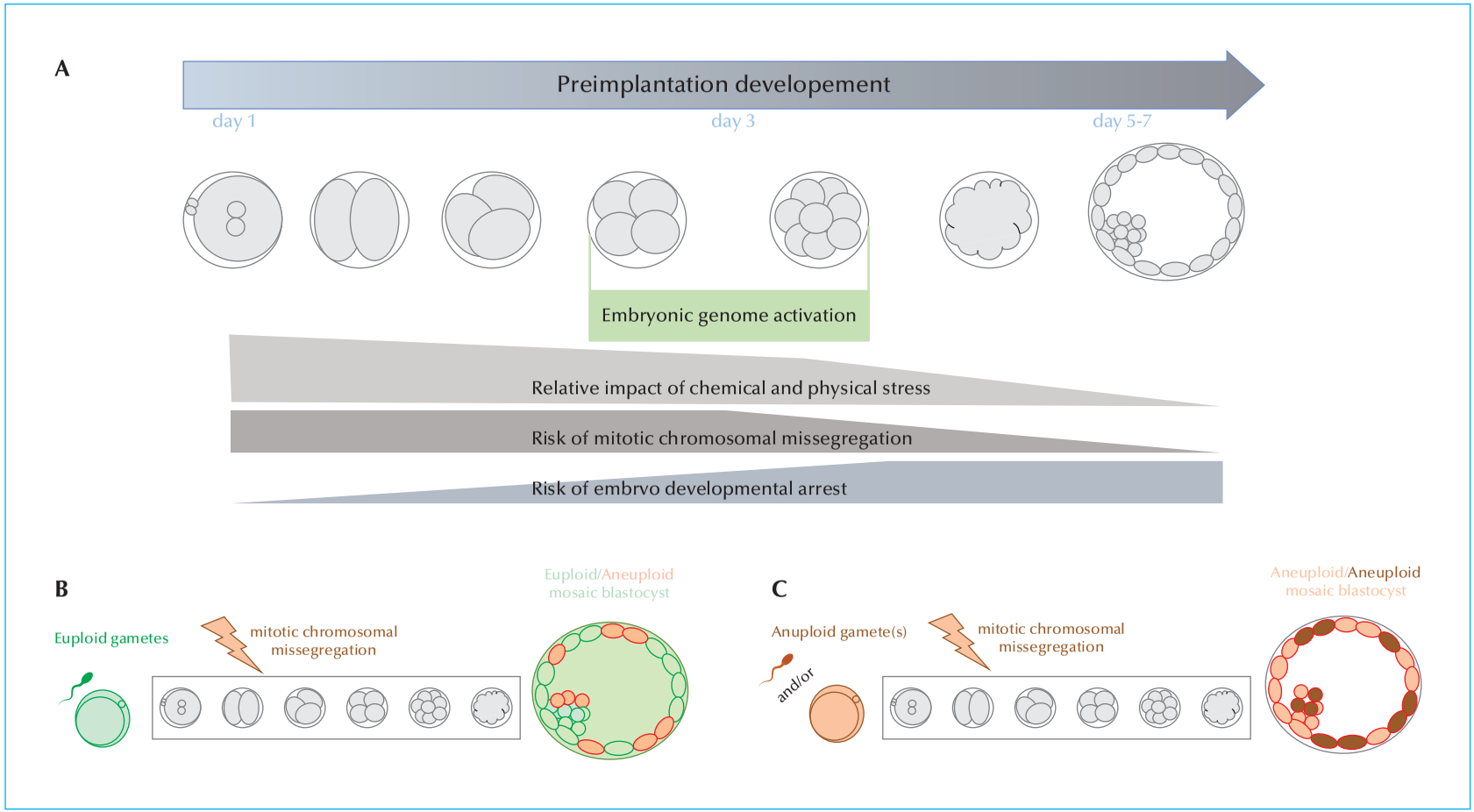

Figure 1. Overview of embryo developmental arrest and chromosomal mosaicism. **A)** The tolerance of the embryo to chemical and physical stress is lower and the risk of mitotic missegregation higher during the early phases of preimplantation development. Nevertheless the risk of developmental arrest is minimal until the activation of the embryonic genome (in human at the 4- to 8-cell transition) when the cell cycle control is finally re-established. Most of the developmentally-incompetent embryos will arrest their development at this stage or in the later ones before the blastocyst stage. **B)** A mitotic chromosomal missegregation occurring in the absence of meiotic aneuploidies in both the gametes might give origin to an euploid-aneuploid mosaic blastocyst. **C)** A mitotic chromosomal missegregation occurring in the presence of meiotic aneuploidies in the oocyte and/or the sperm might give origin to an aneuploid-aneuploid mosaic blastocyst, which does not represent an issue for the reliability of aneuploidy testing.

those countries where embryo biopsy is forbidden and this is the only possible approach. Moreover, PB biopsy, if required, is compatible with a fresh embryo-transfer strategy.

Recently, a multicenter RCT reported that PB-based aneuploidy-testing does not impact embryo reproductive competence and that this approach entails a similar cumulative LBR (CLBR) as standard IVF with higher implantation and lower miscarriage rates, as well as less embryos transferred and vitrified [10]. Nonetheless, its clinical application still shows several limitations: i) the paternal genome and the mitotic errors post-fertilization are excluded from the analysis; ii) the technique is time-consuming and involves a high workload, since all oocytes should be biopsied twice (both PBs are needed), regardless of their developmental competence to blastocyst; iii) it is the least cost-effective approach; iv) it suffers from the same single cell-related issues as for blastomere biopsy; v) low accuracy was reported in predicting the actual embryo chromosomal constitution [11, 12].

All the mentioned reasons contributed in limiting the use of PB biopsy approach, which nowadays is performed only in those countries where it is the only strategy allowed.

Trophectoderm biopsy at the blastocyst stage and related concerns

Blastocyst biopsy entails the removal of 5-10 trophectoderm (TE) cells and their CCT. The higher starting DNA input with respect to single cell analyses *per se* leads to a more reliable genetic diagnosis. Moreover, the TE is the extra-embryonic compartment of the blastocyst that gives origin to the placenta and the other embryonic annexes, while the inner cell mass (ICM), which gives origin to the foetus, is kept untouched.

Blastocyst biopsy methods

Three different blastocyst biopsy approaches have been described: day 3 hatching-based blastocyst biopsy method [13], sequential zona opening and blastocyst biopsy method [14] and day 5/6 hatching-based blastocyst biopsy method [15]. The first protocol is still the most commonly adopted. It entails the laser-assisted zona pellucida drilling at the cleavage stage and extended culture to blastocyst stage for the biopsy. The artificial herniation should involve an easier retrieval of the biopsy fragment, provided that the ICM will not start hatching from the same hole, i.e. a putative drawback of such approach. In addition, the embryo is exposed twice to sub-optimal environmental conditions and laser pulses (to make the hole in day3 and to retrieve the TE cells at the blastocyst stage) and its full expansion is prevented by the presence of the hole. Nonetheless, no RCT to date has ever compared the day3 hatching-based method to the other two blastocyst biopsy approaches. The latter protocols do not involve any manipulation in day3 and the embryo is left undisturbed until the blastocyst stage, avoiding any potential useless source of stress at the cleavage stage. Then, once the blastocyst is obtained and fully-expanded, the day 5/6 hatching-based protocol is more time-consuming and not recommended in a busy PGT unit, differently from the sequential zona opening and TE biopsy protocol which instead involves a higher flexibility for the laboratory in the logistic organization of the daily workload.

TE biopsy is an extensively validated and clinically solid approach; it is considered safe, standardized, informative and cost-effective. A milestone paper in this field is again represented by the non-selection RCT of Scott and colleagues [6], where similar implantation rates were reported between biopsied and sibling non-biopsied untested blastocysts, differently from what outlined for cleavage stage biopsy. Then, several studies conducted in the last few years contributed to the growing worldwide confidence in the implementation of this approach [16-19] (summarized in *figure 2*).

To investigate PGT efficiency, two meta-analyses published in 2015 [20, 21], revealed consistently higher implantation rates per transfer and lower miscarriage when euploid rather than untested blastocysts were replaced in both RCTs and observational studies published along the last decade. The American Society of Reproductive Medicine (ASRM) and the Society of Assisted Reproductive Technologies (SART) recently recognized the clinical value of blastocyst stage PGT, yet outlining the requirement for further investigations on the following pending issues: cost-effectiveness, role and effect of cryopreservation, time to pregnancy, utility in specific subgroups of patients, cumulative success rate over time, total reproductive potential per intervention [22].

Despite all these evidences, TE biopsy is still perceived as the most complicated amongst all biopsy approaches, especially since its application is a multidisciplinary and demanding task. In fact, ICSI, blastocyst culture, vitrification and cryopreserved (single) embryo transfer (ET) must be well-consolidated skills in the laboratory to implement TE biopsy.

Hereafter we reviewed the main criticalities of the techniques indirectly required to efficiently implement a TE biopsy-based PGT strategy (the main advantages and disadvantages of each technique are summarized in *figure 3*).

ICSI-related concerns

A particular concern dealt with the systematic use of ICSI, which is critical for a successful diagnosis during PGT

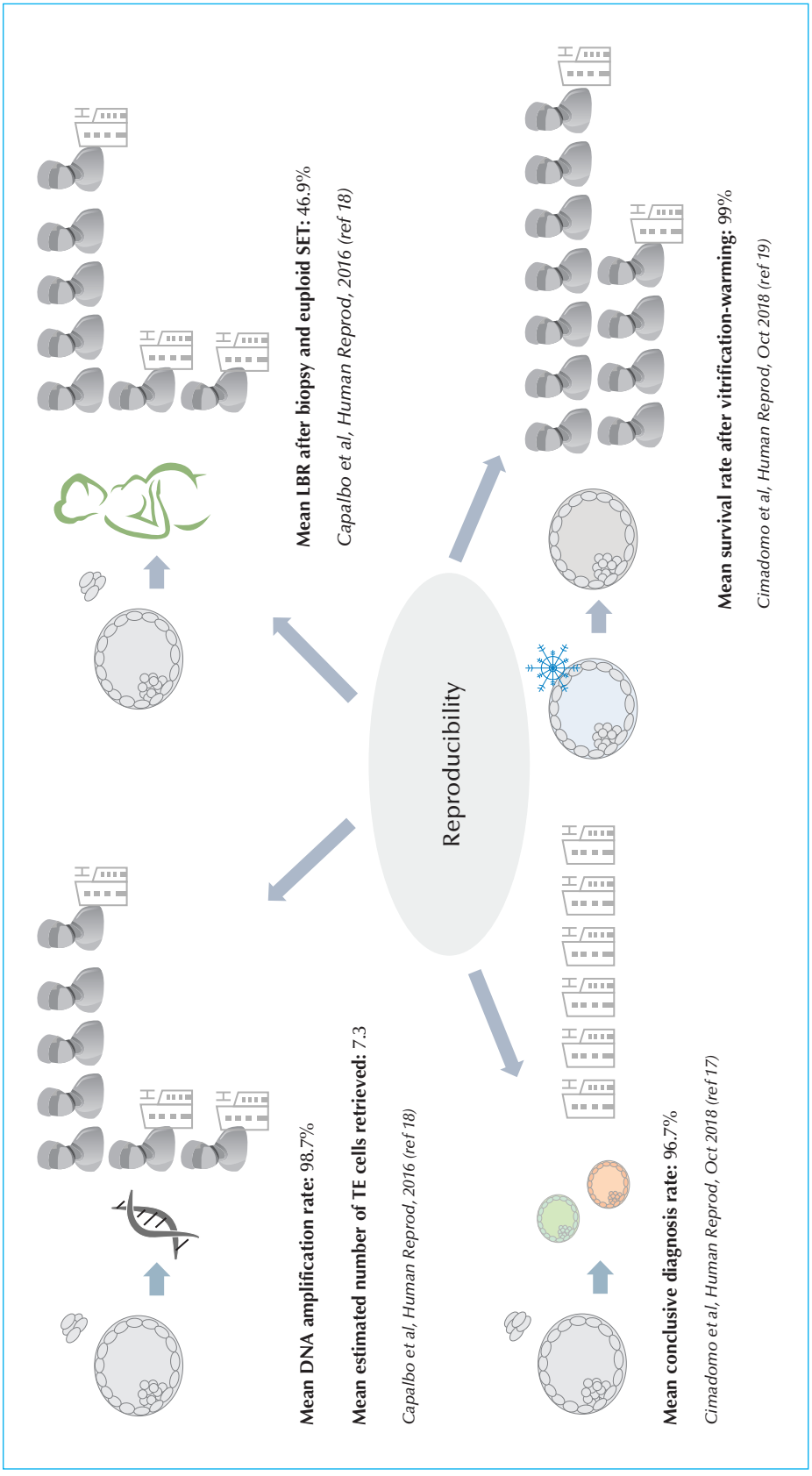

Figure 2. Reproducibility of technical and clinical outcomes of trophectoderm biopsy among several operators from different IVF clinics. *TE*, trophectoderm; LBR, live birth rate.

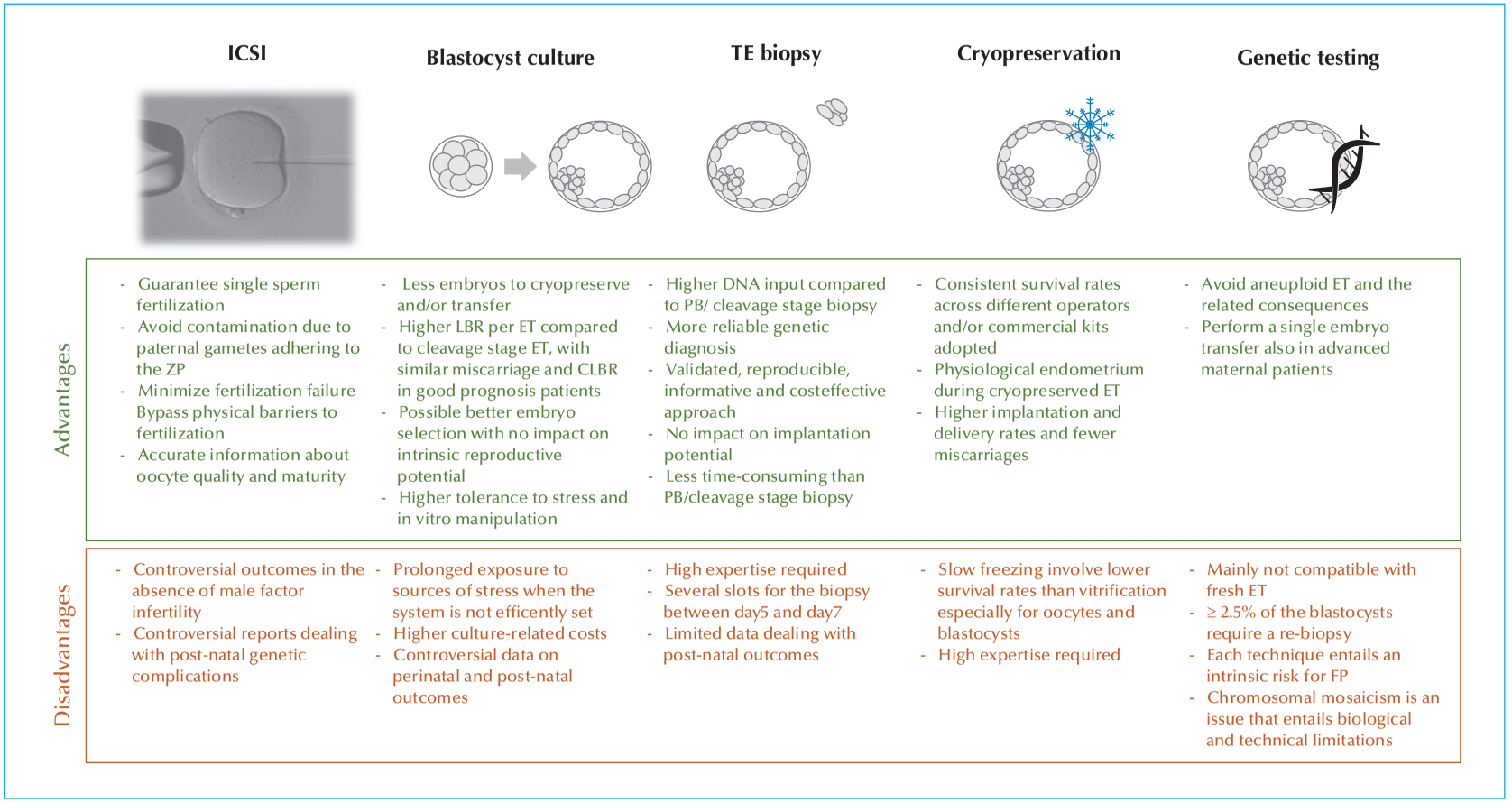

Figure 3. Advantages and disadvantages of all the techniques required for the implementation of blastocyst stage preimplantation genetic testing (PGT). TE, trophectoderm; ZP, zona pellucida; ET, embryo transfer; LBR, live birth rate; CLBR, cumulative LBR; PB, polar body; FP, false positive.

cycles. The rationale is to guarantee a single sperm fertilization, to avoid the potential contamination from extra paternal gametes adhering to the zona pellucida, and to prevent or minimize the risk for fertilization failure (FF).

ICSI was initially introduced in IVF to treat male-factor infertility, but then became common in case of borderline or normal semen parameters. Given the accumulating evidence of its safety and efficacy, ICSI has been widely used in unexplained infertility, poor oocyte quality, low oocyte yield, or AMA to improve fertilization rate (FR) and to reduce total FF. The proponents of such indications for ICSI suggest that its routine use provides more accurate information about oocyte quality and maturity, bypasses any potential barriers to fertilization, and optimizes fertilization outcomes. However, the efficacy of ICSI in non-male factor infertility is still controversial. Some studies reported that the FR and the blastocyst rate were significantly higher in the ICSI group compared to the conventional IVF group, concluding that the fertilization method can be tailored accordingly to improve IVF outcomes [23]. The meta-analysis by Lauren in 2013 [24] supports the use of ICSI to increase FR and decrease the risk of total FF in couples with well-defined unexplained infertility, but also suggested further studies to determine the impact on clinical pregnancy and LBR. In fact, FR and FF are just intermediate outcomes that may not mirror embryo quality and pregnancy potential. In this regard, when setting pregnancy and LBR as primary outcomes, ICSI was reported even less efficient than IVF in the absence of a diagnosis of male factor infertility (SART and CDC. ART success rates: national summary and fertility clinic reports. 1996–2002), as reported also in a large multicenter RCT [25]. Interestingly, the data derived from the National Assisted Reproductive Technology Surveillance System (NASS), based on the information from all ART cycles performed in the USA between 1996-2012, showed that ICSI use doubled across the years and reached up to 76.2% of all fresh IVF cycles. In presence of male factor infertility, the reproductive outcomes including pregnancy, miscarriage and LB rates were comparable to conventional IVF after adjusting for confounders. On the other hand, lower outcomes were reported when ICSI was used in the absence of male factor infertility. These findings suggested that ICSI might improve FR but not implantation or pregnancy rates in case of unexplained infertility, AMA or low oocyte yield [26].

However, Nelson and Lawlor published an outstanding multivariate analysis from the Human Fertilization and Embryology Authority (HFEA) database to investigate all the putative predictors of live birth from the IVF cycles performed in the UK between 2003-2007 (N= 144,018). They reported that ICSI involves significantly higher chance of success. Furthermore, reduced odds for both preterm birth and low birthweight in singleton pregnancies (n = 24,226) derived from ICSI than from conventional IVF [27]. Another concern is indeed represented by post-natal outcomes since studies have shown increased risks for *de novo* chromosomal aberrations [28, 29], major congenital anomalies [30, 31] and imprinting disorders [32, 33] in children conceived after ICSI was used. However, it is still unclear whether the increased risks reported were associated with the use of ICSI or were instead confounded by male-factor infertility *per se*.

In conclusion, still no clear criticism can be moved towards the systematic use of ICSI, since an unbiased evidence of pre or post-natal impact cannot be sustained to date. Therefore, the practice committees of the ASRM and SART did not support the routine use of ICSI in patients without male factor infertility, but could not either exclude its use when required, such as in case of insemination of *in vitro* matured or cryopreserved oocytes, patients with experience of total FF and PGT.

Blastocyst culture-related concerns

The Cochrane review by Glujovsky and colleagues showed that blastocyst culture in good prognosis patients results in a reduced number of embryos available for transfer and/or cryopreservation [34] (i.e. indirectly involving a lower workload for the laboratory). However, despite this reduction, the CLB and miscarriage rates after blastocyst transfer were comparable to cleavage stage ET with significantly higher LBR per transfer (37% versus 29%). In other terms, blastocyst culture involves a better embryo selection without impact on the intrinsic reproductive potential of competent embryos. Then, the straightforward speculation is that, if the culture system is efficiently set, the embryos that do not reach the blastocyst stage are most probably reproductively-incompetent. Nevertheless, extending embryo culture to blastocyst involves a greater and prolonged exposure to different potential sources of stress that must be limited and controlled, as well as higher culture-related costs for the IVF unit. Both chemical and physical factors can affect embryo development *in vitro* as comprehensively reviewed by Wale and Gardner [35], and blastocyst biopsy can be introduced in an IVF laboratory only if these critical prerequisites are fulfilled (summarized in *table 1*).

Among the numerous factors that influence the quality and competence of embryos in IVF, particular attention has been given to the culture media formulation. Different culture media are currently used clinically. The culture strategies can be mainly classified as sequential or continuous. The former strategy involves embryo culture up to the cleavage stage in a first medium to then move it to a different medium up to blastocyst. The rationale behind this change-over step is to mirror the different concentrations

Table 1. Recommendations not to affect embryo development during culture in vitro. VOC, Volatile Organic Compound; CAC, Chemical airborne contaminants

	Air quality	Incubators	Culture	Manipulation
Physical factors	Monitor frequently to assure a range of particles and microbiological parameters within tolerable values Use proper filtering systems Off-gas plastic ware, if required Provide barriers against VOCs	Assure a right number of incubator based on the number of cycles performed Infra-red CO2 sensor incubators Maintain the steadiness of the temperature through a certified thermometer Monitor gas concentration and temperature through calibrated digital carbon dioxide/oxygen analysis units Minimize incubator's door openings	Minimize pH variations around the set point for each specific media Use a proper oil overlay to avoid evaporation and hence prevent the medium from becoming hyperosmotic Minimize the exposure to light Set a correct drop volume and number of embryos per dish	Minimize the number of manipulations to reduce exposure to atmospheric oxygen, light and room temperature Limit the time outside of the incubator Do NOT pipette vigorously Consider the thermal conductivity of the specific dish to calibrate the temperature on warming stages. Prefer working within an isolate/humidified chamber to stabilize temperature, pH and provide a barrier to VOCs
Chemical factors	Provide barriers for CACs that can infiltrate the laboratory, the incubator, and ultimately the culture media. Prefer the use of low odor specialized paints and avoid sealants and toxic glues in the design of the laboratory Positive pressure airflow	Keep the oxygen concentration below 10%	Avoid changes in medium composition in static culture Minimize toxin levels Minimize ammonium other embryo-derived metabolites accumulation	Limit the exposure to hyaluronidase during oocyte denudation Prefer mechanical protocols or the use of a laser rather than chemical treatments to drill the zona and weaken the junctions between embryonic cells

of nutrients between the Fallopian tube and the uterus. The continuous culture strategy instead does not involve any media change-over and, based on the "let the embryo choose" idea, provides all the requirements from day 0. Different studies have compared the two strategies across the years: no effect on LBR was described, just a slightly higher blastocyst rate through some kind of continuous media could be reported [36]. Therefore, if none of the two strategies might be considered better than the other from a clinical perspective, still a continuous approach is possibly more cost-effective and limits the exposure of the embryos to useless chemical and physical sources of stress in day 3 of preimplantation development.

Time lapse incubation has been recently introduced in IVF. This system allows to film embryo preimplantation development within a controlled and undisturbed environment, thereby collecting the related timings of cell division and inspect putative predictive algorithms. Nonetheless, time lapse developmental parameters showed very limited predictive power to conduct embryo selection, especially in a setting already involving blastocyst culture and aneuploidy testing [37]. In fact, no increased clinical outcomes with respect to conventional incubation have been reported to date [38]. Still, if the system is already in-house, it guarantees undisturbed culture conditions, as well as a deeper investigation of embryo fertilization and developmental events, which are important advances that should not be disregarded.

While no consensus has been reached regarding the clinical usefulness of time lapse parameters, the controlled and undisturbed nature of embryo culture conducted in time lapse incubators has pointed out the detrimental and irreversible impact of atmospheric oxygen on embryo development [35]. In fact, an atmosphere rich in oxygen generates a greater quantity of reactive oxygen species (ROS) which are in turn responsible for affecting cell function at several levels (e.g. structure of membranes and organelles, damaging DNA and altering genetic expression) [35]. The importance of a low oxygen concentration is particularly crucial when culturing embryos up to the

blastocyst stage, possibly since ROS production gradually increases at advanced stages of development. However, a negative influence cannot be excluded even if culture is limited to day 2 or day3 of development. In fact, the first and to our knowledge only Cochrane review investigating the LBR according to oxygen concentration showed a significant increase in the LBR in the low oxygen arm [39], as confirmed also from a more recent meta-analysis published in 2016 [40].

A last concern is related with obstetrical and neonatal outcomes after blastocyst transfer. Also, when addressing this issue, controversial data were published, possibly due to different study selection parameters, patient population and IVF laboratory settings (e.g. fresh or cryopreserved ET). Some studies reported higher risk for preterm birth, perinatal mortality and placental complications with blastocyst rather than cleavage stage ET [41, 42]. However, such result was reported only when singleton pregnancies achieved after fresh transfer were accounted and could not be confirmed when cryopreserved transfers were instead inspected [43]. The inspection of cryopreserved blastocyst transfer highlighted only a higher risk of infants large for gestational age, a condition certainly less alarming. Then, further criticisms were also recently moved to the claims of worse obstetrical and neonatal outcomes derived from blastocyst transfer. In particular, the systematic review and meta-analysis by Martins and colleagues, excluded significant differences regarding the incidence of birth defects or low birth weight between blastocyst and cleavage stage transfers, while the risk for small for gestational age seemed even higher with the latter strategy [44]. Finally, also embryo culture conditions should be taken in account. In fact, while the studies reporting adverse effects by performing extensive cultures in vitro seem to have in common the use of 20% oxygen tension [45], the studies using instead low oxygen tension do not report any adverse effect on the children born following extended culture [46, 47].

To conclude, perinatal and postnatal outcomes clearly need to be monitored in the next years to shed more light on this still unclear topic. A clear evidence might be produced only if putative confounders (e.g. fresh/cryopreserved transfer, high/low oxygen tension) will be accounted in the investigation.

Embryo cryopreservation-related concerns

In order to implement blastocyst stage biopsy in an IVF unit, a validated cryopreservation system is mandatory. The implementation of cryopreserved ET strategy has grown across the last years. In Europe in 2011 the cryopreserved transfer cycles represented 32% of the procedures performed, a rate that in northern European countries (i.e. Switzerland, Finland, Netherlands, Sweden and Iceland) even exceeded 50% [48]. These numbers are imputable to an increased implementation of strategies such as cycle segmentation (i.e. cryopreservation after ovarian stimulation and transfer on a non-stimulated endometrium in a following menstrual cycle), oocyte banking for medical or non-medical reasons and PGT.

Numerous cryopreservation protocols have been introduced with different type and concentration of cryoprotectants, equilibration timing, cooling rates and cryopreservation devices used. However, all of them can be classified as representative of either slow-freezing or vitrification approach [49]. According to the former approach (equilibrium freezing), the cells or tissues are equilibrated in a low concentration of cryoprotectants, then with the support of a programmable freezing machine, they are exposed to a slow decrease in temperature until the final transfer into liquid nitrogen for storage. Vitrification (non-equilibrium protocol) instead combines the use of high concentration of cryoprotectants with ultra-rapid cooling rates involved by the direct contact of the cells or tissues with liquid nitrogen: an amorphous glassy solid is formed which prevents from the formation of ice crystals.

The use of one technique over another depends on the type of biological material that must be cryopreserved. Vitrification has been agreed as the best method for blastocysts and oocytes with survival rates as high as 96-98%, while comparable results might be achieved with cleavage stage embryos (94%) with the two cryopreservation protocols. These data have been systematically reviewed in the meta-analysis by Rienzi and colleagues [50], which also highlighted how vitrification is becoming more widespread in the IVF practice worldwide, especially since it has been recognized a safe clinical approach by the ASRM and the SART [51] involving high CLBR.

Recently, the systematic application of vitrification in two experienced IVF centers resulted in survival rates even higher than 99% if a collapsed blastocyst was cryopreserved both after laser-assisted artificial shrinkage during conventional IVF cycles or after TE biopsy during PGT ones [19]. These rates were even significantly higher than what reported for non-biopsied and non-collapsed blastocysts during conventional IVF cycles (ca. 97%). An evidence that further supports the high resistance of human blastocysts to embryo manipulation which, for some instances, can even be beneficial. From a procedural perspective, the survival rates were consistent across different vitrification and warming operators and/or commercial kits adopted [19]. Lastly, we recently reported that in case of inconclusive diagnoses involving the need for a second CCT analysis, a further TE biopsy and vitrification-warming cycle did not seem to impact embryo reproductive potential [17, 52]. All these evidences together support the reliability of a setting which entails blastocyst biopsy and

vitrification in the hands of expert operators and equipped IVF units.

Fresh or cryopreserved embryo transfer?

Fresh and cryopreserved ET could result in similar delivery rates [53], but the latter might be safer in terms of obstetrical and perinatal outcomes (i.e. less likely perinatal mortality, small for gestational age, preterm birth, low birthweight and hemorrhage) [54]. Nevertheless, some authors claimed that also higher delivery rates are involved from cryopreserved ET [55]. Accordingly, some evidences demonstrate that the endometrial receptivity might be impaired in fresh transfer cycles due to a putative negative effect of the ovarian stimulation on endometrial transcription and angiogenesis [56]. Therefore, a freeze-all approach (regardless the adoption of PGT or any other strategy of embryo selection) was suggested not only to minimize the risk of ovarian hyperstimulation syndrome but also to increase the chance of implantation. In conclusion, if no clear statement might be done to support that a freeze-all strategy is better than a fresh transfer approach, however the former is certainly not to be discouraged when strictly required, as for PGT purposes.

Chromosomal mosaicism and related concerns

Chromosomal mosaicism is a phenomenon represented by karyotypically-different cell lines in the same embryo. These intercellular differences in chromosome content is a consequence of post-zygotic chromosomal missegregation. Mitotic aneuploidies can occur at all preimplantation stages and seem independent from maternal age [4], differently from meiotic constitutive (uniformly present in all cells) ones [1]. If a mitotic error occurs in an embryo which was already aneuploid because of a pre-existing meiotic impairment, the embryo will be an aneuploid-aneuploid mosaic, which does not represent an issue for PGT since the embryo will be classified aneuploid in any case. On the contrary, a mitotic error in the absence of meiotic ones originates an euploid-aneuploid mosaic embryo, which represents a hazard for a correct diagnosis.

Chromosomal mosaicism: general considerations

While the reproductive consequences of full-chromosome meiotic aneuploidies are well-defined, when it comes to mitotic aneuploidies they might vary according to many variables like the chromosome involved, the developmental stage of missegregation occurrence (the earlier the mitotic error, the greater the prevalence of mosaicism and vice versa), the location of the cells involved [57-59].

Detecting mosaicism in human embryos is a challenging task. Indeed, several studies reported extremely variable estimates [60, 61]. The highest evidence of mosaicism in an embryo is represented by reciprocal aneuploidies (e.g. trisomy-monosomy for the same chromosome) reported by a double biopsy and blinded analysis. Currently, mosaicism is instead reported clinically based on a single TE biopsy and the fluctuation of the copy number profile for a (or several) specific chromosome(s) [62]. This represents *per se* a limitation to a proper detection, since this framework might suffer from biological, technical and methodological issues. For instance, the attempt of reporting mosaicism from a single 5-10 cells biopsy involves an inevitable sampling bias, implicit to the definition of mosaicism itself.

Detecting mosaicism at the cleavage stage

Even if the inactivation of cell cycle control in the early cell divisions along preimplantation development makes mitotic segregation error-prone [3, 4] and mosaicism more likely at the cleavage stage, an accurate estimate of its prevalence from blastomeres is tricky. In fact, the analysis of normal blastomeres could frequently involve a false positive diagnosis. Therefore, when multiple blastomeres retrieved from the same embryo at the cleavage stage are analysed in separate reactions by CCT platforms (e.g. array-CGH), even a single artefact is sufficient to erroneously classify that embryo as mosaic [62-64]. At last, mosaicism cannot be detected in a clinical setting at the cleavage stage. In fact, this approach entails the CCT of just one cell.

Detecting mosaicism at the blastocyst stage

The multicellular nature of TE biopsies allows an improved chance to estimate mosaicism from both a basic research and a clinical workflow [2, 62, 65]. Indeed, several published studies have reported the clinical implementation of CCT technologies to estimate mosaicism at the blastocyst stage from a biopsy (e.g. [66, 67]).

However, attempting to "diagnose" mosaicism entails biological and technical limitations that cannot be disregarded. In fact, even if PGT technologies have been perfected to minimize errors and optimize the diagnosis, full accuracy is utopian and the diagnosis will always face some artefacts and limitations that need to be acknowledged to both the practitioners and the patients [2, 62, 65, 68]. Moreover, the more the sensibility of a diagnostic technique is pushed, the more its specificity might be reduced [69]. This is especially true if, as in the case of the NGS-based "diagnosis" of mosaicism, no

further molecular procedure are involved, but simply the customization of downstream bioinformatic parameters to inspect the data [70]. The "diagnosis" of mosaicism is indeed based on two premises: i) the fluctuation of the copy number profile of a specific chromosome in the middle between the disomy and aneuploidy thresholds pictured in the CCT plot might derive from a biopsy composed on both euploid and aneuploid cells, ii) the biopsy containing both euploid and aneuploid cells might be representative of the same condition in the rest of the TE and the ICM. In other terms, a "diagnosis" of mosaicism is conceptually unrealistic, while a "prediction" of the chance that an embryo is mosaic better describes the attempt of reporting clinically such condition based on the CCT of few TE cells. Hereafter, we reviewed the putative sources of biological and technical artefacts.

Putative biological artefacts

– An equal number of TE cells carrying a reciprocal aneuploidy (e.g. monosomy-trisomy for the same chromosome) due to a mitotic nondisjunction event might result in an euploid plot since the DNA missing from some cells will be compensated by the additional DNA from the other cells. In this case a mosaic blastocyst could be erroneously diagnosed as euploid.

– Polyploid embryos (e.g. trisomic) with an extra (e.g. tetrasomy) or missing (e.g. disomy) chromosome can be reported as mosaic.

– A TE biopsy with a significant presence of cells in the S-phase of the cell cycle can result in a copy number profile erroneously indicative of mosaicism.

Putative technical artefacts

Few TE cells are not sufficient to provide enough starting DNA input to perform the analysis, therefore a DNA enrichment step (pre-amplification) is required which might introduce a bias. Either an over-amplification of a specific chromosome possibly resulting in an euploid embryo "diagnosed" as mosaic, or an under-amplification of a specific chromosome possibly resulting in an aneuploid embryo "diagnosed" as mosaic.

DNA contamination: an aneuploid biopsy could be contaminated by euploid cells from the operator(s) involved in the biopsy and/or molecular analysis procedures. In this case the chromosome copy number from the biopsy is lowered and an aneuploid embryo might be erroneously reported as mosaic.

Clinical considerations and future perspectives

The most reliable estimate of the prevalence of mosaicism at the blastocyst stage derives from research papers where donated human embryos were disaggregated in different sections of the whole TE and related ICM, then analysed separately [71-77]. If accounting only full chromosome aneuploidies, the prevalence of euploid-aneuploid mosaicism did not exceed 5-6% of the blastocysts analysed (overall more than 400 to date). In contrast, if the analysis is performed on a single TE biopsy, the reported prevalence of euploid-aneuploid chromosomal mosaicism raises up to more than 14% (even more than 20% if including also mosaic segmental aneuploidies; overall more than 29,000 blastocysts analysed) [78]. The gap among the prevalence reported from research and clinical studies might be imputed to the biological and technical artefacts listed above and their impact on the reliability of "diagnosis" of mosaicism based on few TE cells. A 5-6% prevalence of euploid-aneuploid mosaic blastocysts is more reasonable even when compared to the rates reported from prenatal diagnosis. Mosaicism is lower than 2% from both spontaneous and IVF-derived pregnancies diagnosed after chorionic villus sampling, where true foetal (not placental confined) mosaicism confirmed via amniocentesis is even lower than 0.5% [79].

Counselling a couple that receives a PGT report identifying a blastocyst as mosaic based on a single TE biopsy is still chancy at present. The IVF practitioners and genetic consultants in fact cannot yet estimate the clinical positive and negative predictive values underlying the transfer of a "mosaic" blastocyst, nor the inherent reproductive hazards are foreseeable. The answers to this controversy might be produced only through non-selection studies where "mosaic" blastocysts must be transferred blindly to both the clinician and the couple. Only after the clinical outcome would be established, the plots should be unblinded to outline the chance that a blastocyst reported mosaic after CCT would have implanted, involved a miscarriage or an implantation failure. This design was adopted back in 2012 by Scott and colleagues to define the positive and negative predictive value of SNP-array-based CCT on TE biopsies. This study, where only constitutive full chromosome aneuploidies were accounted, represents still nowadays the main landmark paper for PGT-related counselling [80].

Conclusions

Across the years, the introduction of systematic ICSI, blastocyst culture, freeze-all strategy, SET as well as an increasing application of PGT in AMA patients in our clinic did not affect the CLBR with respect to a previous framework mainly entailing fresh double ET at the cleavage stage, while significantly reducing the miscarriage and multiple pregnancy rates [81]. These results clearly demonstrate that the technological advances in IVF, if conscientiously implemented after a strict validation, applied in couples with the proper indications and constantly monitored for their performance, are not harmful for the embryos and might lead to a higher efficiency

Table 2. Key performance indicators involved by blastocyst stage preimplantation genetic testing. Basic competency and benchmark values representing the minimal and the ideal outcomes achievable by performing IVF procedures entailed by preimplantation genetic testing. A constant monitoring of these indicators in each unit is required to evaluate the laboratory skills as part of the quality management system. Adapted from the Vienna Consensus: report of an expert meeting on the development of ART laboratory performance indicators (2017). MII, metaphase II; PN, pronucleus; PB, polar body; SET, single embryo transfer.

	Indicator	Calculation	Competency value	Benchmark Value
Insemination	ICSI damage rate	**no. damaged or degenerated oocytes / no. injected oocytes**	≤10%	≤5%
Fertilization	ICSI normal fertilization rate	**no. oocytes with 2PN and 2PB / no. oocytes injected**	≥65%	≥80%
Developmental rate	Blastocyst development rate	**no. blastocysts obtained / no. oocytes with 2PN and 2PB**	≥40%	≥60%
Biopsy	Successful biopsy rate	**no. biopsies with a conclusive diagnosis / no. blastocysts biopsied**	≥90%	≥95%
Cryopreservation	Blastocyst cryo-survival rate	**no. blastocysts survived / no. blastocysts warmed**	≥90%	≥99%

and safety of the treatment. Importantly, human blastocyst seems more resistant to all putative sources of stress than the oocyte or the cleavage stage embryo. In this regard, any IVF-related manipulation, like zona pellucida drilling, biopsy and vitrification might be more tolerated at the latest stage of preimplantation development than during the earlier ones (*table 2* summarizes the competency and benchmark values of the key performance indicators needed for blastocyst stage PGT).

The future in this field might involve a further reduction of the costs of genetic testing, as well as bring about a revolutionary PGT framework based on the analysis of spent culture media as a totally non-invasive source of embryonic DNA. The latter, if scrupulously set and validated in the next years, is expected to be a game-changer approach in IVF since it will allow more clinics and patients to access the benefits of PGT with even less putative methodological drawbacks.

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